Original Paper
ISSN: 2321-1520

Isolation and Characterization of Pectin Degrading Bacteria from Peels of Rotten Simple and Accessory Fruits

Priyanshi R. Rami[1*], Megha R. Makwana[2] and Tisha Patel[3]

Department of Microbiology and Biotechnology, School of Sciences,
Gujarat University, Ahmedabad.
E-mail : priyanshirami1995@gmail.com

Note: This Paper was presented at National Symposium held at the Department of Microbiology & Biotechnology. It won the best Paper/Poster Award.

Received Date: 20-4-2017
Published Date: 15-9-2017

Abstract

Pectinases are a big group of enzymes that break down pectin polysaccharides from plant tissues into simpler molecules like galacturonic acid. It has long been used to increase yields and clarity of fruit juices. In this study, five bacterial strains were isolated from rotten fruits (orange, banana and apple) using dilutions 10^{-2} to 10^{-4} on 2% pectin agar medium (PAM). Isolated organisms were identified on the basis of staining and biochemical tests. The pectinolytic activity was determined using pectin containing minimal essential medium and carrot waste medium (CWM). The methodology applied was plate assay at the temperature 30 ± 2p C. All the five strains showed different pectinolytic zones depending on the concentration of the inoculums and the largest pectinolytic zone of 11mm was observed from the isolate obtained from apple fruit. The above isolates can prove to be efficient and promising production of pectinase. Also the 2 isolates which are showing more enzyme activity used for the application part like clearance of fruit juices (Orange & Apple)

Keywords: Pectin, Pectinase, rotten fruits (orange and apple), CWM.

Introduction

Pectinases are a big group of enzymes that break down pectin polysaccharides from plant tissues into simple molecules like galacturonic acid and the sugars. Chemically pectic substances are complex acid polysaccharides. The side chains consists of L-rhamnose, arabinose, galactose and xylose, most of the sugars present in pectin molecule are reducing in nature (Be Miller, 1986).

On the basis of modification of the backbone chain pectic substances are classified into protopectin, pectic acid, pectinic acid and pectin (Be Miller, 1986). Pectinases most commonly causes the breakdown of α (1-4) glycosidic linkage between the galacturonic acid molecules. Pectinases are produced during the natural ripening process of some fruit and act in combination with cellulases (Jabeen A et al., 2015).Pectinases were some of the first enzymes to be used in homes. Their commercial application was first observed in 1930 for the preparation of wines and fruit juices (Oslen, 2000). Pectinases is a general term used for a mixture of several different enzymes which break down pectin molecules. Pectinases are responsible for the degradation of the complex molecules in the fruit pulp called pectin and responsible for turbidity in pulp (Tapre, A.R and Jain, R.K., 2013). With the addition of Pectinases the jelly structure disintegrates so the press ability of the pulp increase and the fruit juice is easily obtained with higher yields. Pectinases are inducible and they can produce by using different carbon sources. Pectin degrading enzymes have been extensively used at the industrial level to improve the stability of fruit and vegetable juices and in wine industries also Pectinases are used (Tressler and Joslyn, 1971). The pectinolytic enzymes are also used in sugar extraction process from date fruits (Bahramian et al., 2011), DE skinning of orange segments, wine clarification was first recommended by Bensone and Cruess (1941), to enhance the oil extraction from vegetables (Hankin L et, al., 1999), for making vegetable purees. Mainly the pectinolytic enzymes are produced from many microorganisms like bacteria, yeast and a large number of fungi, most relevant ones are Aspergilus sp (Jabeen A et al.,2015). The selection of high yielding pectinase producing strain is difficult. However studies revealed that fungal ones show to produce less yield of pectinolytic enzymes than bacterial strains (Suneetha et al., 2011). The objective of the present study is to isolate the bacteria which have ability to produce pectin degrading enzyme from fruit waste and peels and their characterization. To check the activity of selected isolates for pectinase enzyme production by DNSA method and application of the produced crude enzyme for clarification of fruit juices.

Materials & Methods

Sample collection

For the isolation of pectinase producing bacterial strain, partially decayed fruits (Orange, Apple & Banana) were taken and stored in sterile polythene bags from the local market Manekchawk, Ahmedabad (23.0225° N, 72.5714° E). Till further proceedings, samples were stored in freeze around 5-10ºC.

Isolation and identification of pectin degrading bacteria

Rotten apple, orange, and Banana were used to isolate pectin degrading bacteria. The rotten fruit parts were scrapped by using sterile forceps and serial dilutions (10^{-1} to 10^{-6}) were made in sterile distilled water. A total of 0.2 ml of each dilution was spread on 2% pectin agar plates (pH around 4.5). These plates were incubated for 24 to 48 hours at 28±30ºC. The bacterial colonies appeared on plates were further purified on same medium under the same conditions. Bacterial isolates showing good Cx ratio were identified on the basis of colony morphology (Size, Shape, Elevation, Texture, margin, Opacity, Consistency and Pigment) (Table 1), Gram Staining and biochemical testing by using Hi-media biochemical kit (Hi-media, USA).

Characterization of pectin degrading bacteria

Selected isolates were further screened for its pectinase producing ability. The selected pure isolates were again inoculated on the same 2% pectin agar medium. And after the incubation the plates were flooded with 2% Iodine solution and incubated for 15 min at 37ºC. A clear zone around the growth of the bacterial colony was indicated to pectinase activity. These isolates are characterized on the basis of their colony characters and Gram staining. Cx ratio was calculated according to the formula given by Guangyi et al (1996).

Cx ratio = Diameter of zone of clearance / Diameter of colony.

Production medium for pectinolytic enzyme

Enzyme assay was carried out by using carrot waste medium (CWM) and synthetic minimal medium. In 250ml Erlenmeyer flasks, 20g of crushed carrot waste was added and dispensed with 100ml of Distilled water. To this flask, 1ml activated bacterial suspension from each LB broth was added and one flask was kept as a control. Inoculated flasks were incubated on a shaker at 120 rpm for 12 hours and the controls were kept at room temperature. After that the supernatant was removed from medium by centrifuging at 6000rpm for 20 min and the clear filtrate was used for determining pectinase activity. For minimal medium, flask were prepared using pectin containing 100mL minimal essential medium (pH 5) composed of 0.05% KCL, 0.1% $MgSO_4$ 0.1%$MgSO_4 \cdot 7H_2O$, 0.1% Trisodium citrate dihydrate, 0.1% citric acid, 0.1% yeast extract, 0.1% casein (acid hydro lysate), and 1.0% pectin pure(HPLC Lab reagents) (Ranveer Singh Jayani et al.,2010). 1ml fresh inoculum was added in flasks and one was kept as a control. All Inoculated flasks were incubated on a shaker at 120 rpm for 12 hours and the controls were kept at room temperature. The supernatant was removed from cultural broth by centrifuging at 6000 rpm for 20 min. This clear filtrate was further used for determining pectinase activity.

Enzyme Assay

Pectinase activity was assayed by the colori-metric method of (Miller, 1959). Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNSA) method. For this, to 1 ml of 1% pectin containing sodium citrate buffer of pH 5.0 and 0.5 ml of enzyme extract was added. The reaction mixture was incubated at 35°C±1°C for 20 min After 20 min, 1.5 ml of DNS reagent was added and the test-tubes were shaken to mix the contents. The test-tubes were heated to boiling on the boiling water-bath for 10–15 min. Then these were cooled and 7 ml of distilled water was added to the contents of each tube and the absorbance was measured at 540 nm using Spectrophotometer. The enzyme and substrate blanks were run parallel. The standard curve was prepared for reducing sugars with galactose. One enzyme unit of polygalacturonase is the number of µM of reducing sugars measured in terms of galactose, produced as a result of the action of 0.5 ml of enzyme extract in 1 minute at 35°C ± 1°C (figure 3, 4).

Application of crude pectinase in juice extraction and clarification

Fruit juices are turbid and cloudy after their extraction from pulp. For the clarification of

juices enzymes were used at industrial level. Use of pectinase enzyme is very common for clearance of juices. Pectinase enzyme used in combination with cellulases and hemicellulases enzymes for clarification. The pectinase enzyme was extracted from pectin minimal medium (3 days, 160 rpm, 30±2ºC). Then these pectinase enzymes were added to the unclarified juice of orange and apple in 250 ml flask. The flasks containing the enzyme and the juice were kept for 2 days at 120 rpm. The flasks were then kept in the water bath for 1 hour at 90 ±2ºC. The juice was then filtered using whattman filter paper. This juice was then checked for its viscosity by Ostwald's viscometer. The control was also kept which contained no enzyme.

Results

Isolation and Screening

Total Eight bacterial isolates were obtained during isolation from (onion, orange, apple and banana) on the 2% pectin agar plate and they were named as Oni1, Oni2, Ora, App, B1, B2, B3. From them five isolates isolated from Orange, Apple and Banana showed translucent clear zone around the colony on addition of 2% iodine solution and were taken in to consideration for further process. The Cx ratios was calculated to estimate the amount of enzyme produced by the selected isolates by the formula given in.

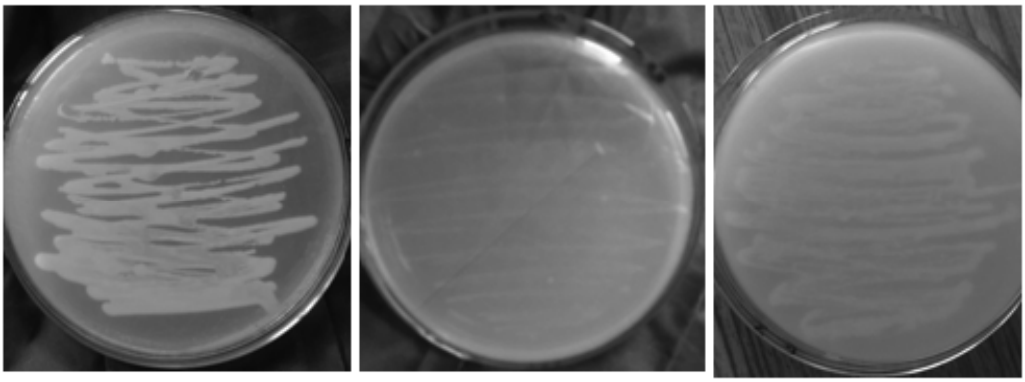
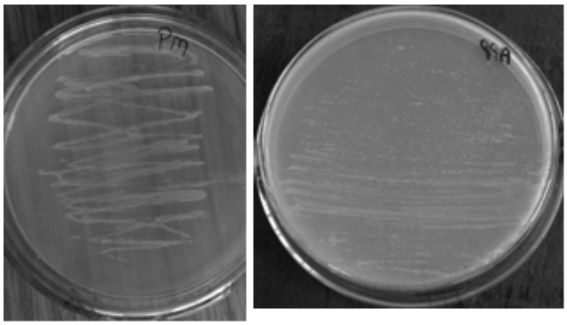


Figure: 1. [A] [B] [C]

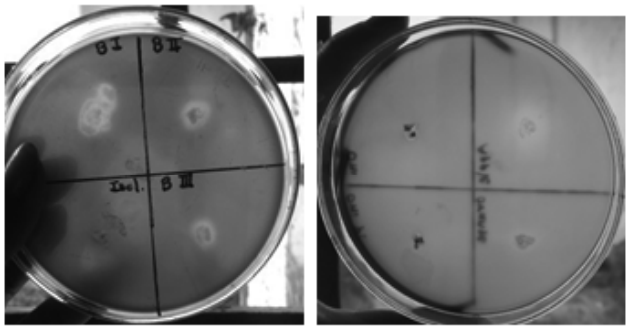


[C] [D]

[Fig: 1: Isolated pure bacterial culture which has showed a clear zones around colonies. Where A=B1, B=B2, C=B3, D=Ora, E=App]

Table: 1 Colony characteristics and Gram staining of the bacterial isolates which shows clear zone on plates.

Isolates	Size	Shape	Elevation	Texture	Margin	Opacity	Consistency	Pigment	Gram's Reaction
B1	Intermediate	Round	Raised	Rough	Entire	Opaque	Dry	Nil	Gram positive Bacilli
B2	Small	Round	Raised	Smooth	Entire	Translucent	Moist	Nil	Gram negative short rods
B3	Small	Round	Slightly raised	Smooth	Entire	Translucent	Moist	Nil	Gram negative short rods
Ora	Intermediate	Round	Slightly raised	Smooth	Entire	Translucent	Moist	Nil	Gram negative short rods
App	Small	Round	Raised	Smooth	Entire	Transparent	Moist	Nil	Gram positive Cocci



Characterization by plate assay

For the screening of pectin degrading isolates, they were tested on the pectin agar plates to check their zone of clearance was measured (figure 2).

Fig: 2 Zones of pectin degradation on 2% pectin agar plates flooded with iodine. The zones were observed for isolates B1, B2, B3, Ora, and App. Highest zone was observed by B3 and App isolates.

Table: 2 The Cx ratio of all the five isolates.

Cx ratio = Diameter of zone of clearance / Diameter of colony.

Isolates	Zone of Clearance (mm)	Cx Ratio
B1	6	2
B2	8	2
B3	9	2.25
Ora	9	1.8
App	11	2.75

Highest Cx ratio was observed by B3 and App isolates.

Enzyme Assay

Enzyme production was checked on both carrot waste medium as well as synthetic minimal medium for the better production of pectinase enzyme

Table: 3.1 Results of enzyme assay in carrot waste medium.

Isolates	Enzyme activity (U/mL) at			
	12 hours	18 hours	24 hours	48 hours
B1	2.05	4.11	5.44	8.88
B2	2.22	4.05	5.33	7.61
B3	3.11	7.05	7.16	8.33
Ora	3.05	6.33	6.66	7.38
App	3.22	7.33	8	9.33

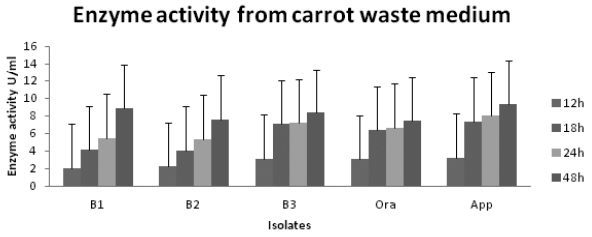


Figure 3: Shows the isolate App (apple) showed higher enzyme activity after 48 hours.

(App showed 9.33 U/ml of enzyme production)

Table 3.2: Results of enzyme assay in synthetic medium.

Isolates	Enzyme activity (U/ml) at			
	12 hours	18 hours	24 hours	48 hours
B1	3.66	4.55	6.67	10.22
B2	3.77	4.27	5.38	9.5
B3	5.61	6.66	8.61	10.56
Ora	3.88	5.56	6.22	9.22
App	6.77	6.89	8.61	10.66

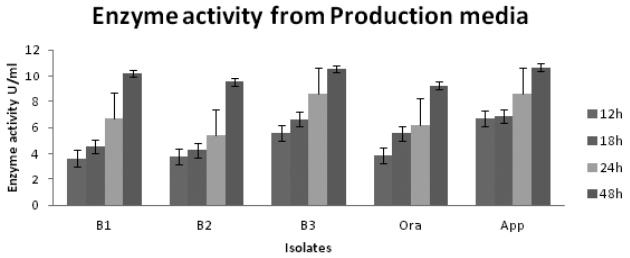


Figure 4: Shows the isolate B3 and App showed higher enzyme activity after 48 hours. (B3 showed 10.56 U/ml and App showed 10.66 U/ml of enzyme production)

Application of crude pectinase enzyme

From the production medium of the isolates B3 and App, enzyme was extracted by centrifugation and then used for the clarification of orange and apple juices and their viscosity was measured using Ostwald's viscometer.

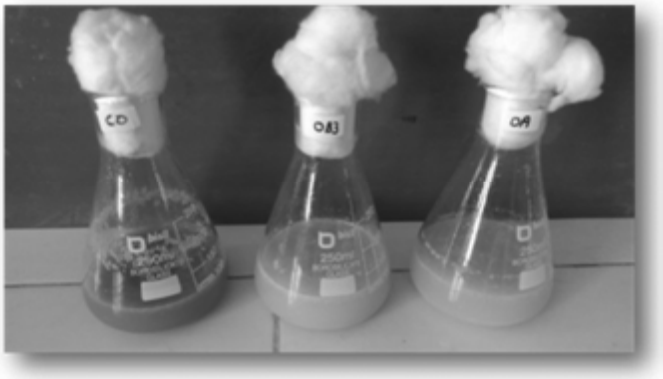


Fig 5: Clearance of Orange juice on the addition of bacterial crude enzyme after 48 hours as compared to the control (without enzyme).

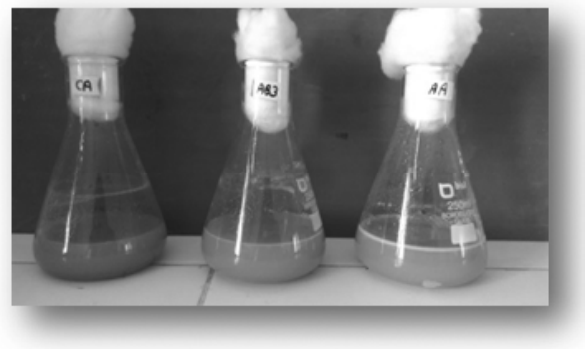


Fig 6: clearance of Apple juice on the addition of bacterial crude enzyme after 48 hours as compared to the control (without enzyme).

Viscosity Measurement

Table 4: Viscosity measurement of the juice by Ostwald's viscometer.

	Orange juice(time in sec)	Apple juice (time in sec)
Control	4.3	4.8
B3	3.3	4.4
App	3.1	4.0

The enzyme treated juice shows less viscosity than control (without enzyme).

Discussion

In the cell walls of fruits and vegetables, pectin is the predominant component (Anuradha et al., 2010) which supports the growth of pectinolytic isolates. Hence vegetables and fruits are good sources of pectinolytic enzymes. E. Venkata et al (2013) searched many promising strains of pectinase producer is a continuous process. In Hankins medium investigators reported maximum enzyme activity at pH 7.0 which was significantly higher than one found in the present study (pH 6.0). Kumar and Sharma (2012) isolated pectinase producing Cocci from decomposing fruit materials and screened for their pectinolytic activity after 72 hours of incubation time maximum enzyme activity 13.96 U/ml was obtained. In our case, App isolate from the decayed Apple was identified as Cocci by Gram staining which showed the highest zone of clearance around the colony and the enzyme activity (10.66 U/ml) was observed after 48 hours in synthetic medium. Also isolate B3 from rotten Banana identified as short rod which showed second higher enzyme activity (10.56 U/ml) compare to App isolate. Albersheim and Killias (1962), Bateman and Miller (1966) and Moran *et al.* (1968) reported that most of the organisms showed pectinolytic activity at pH 6, while few exhibiting at 6.5 and 5.5. The ability of the organisms to grow best and mostly at pH 6 indicated that greater portion of the enzymes produced by the test organisms are polygalacturonase. In this study, the isolates were obtained near about pH 5 to 5.5. Although many fungi and yeast (Birgisson et al., 2003) produced exopolygalacturase, both are slow growing

and processing microorganisms. Thus, this study was aimed at screening fast growing and processing bacteria which produce high level of polygalacturonase. The results obtained in the present study indicated that this newly extracted bacterial isolates can also be further exploited for the pectinase production by using varying mechanisms and applied for their significant applications.

Conclusion

From this study, we isolated total eight isolates from which five isolates were further selected to determine its pectinase enzyme activity on the basis of their clear zone around the colonies. Isolate B3- Gram negative, short rod and isolate App - Gram positive Cocci were isolated from the rotten banana and apple respectively with highest enzyme activity (B3 showed 10.56 U/ml and App showed 10.66U/ml) and were found as efficient pectinase producing bacterial strain that can be used for various industrial applications. Extracted pectinase enzyme was used in the clarification of juice and it was observed that the viscosity of the enzyme treated juice was decrease because of the degradation of pulp in it. So, the present investigation reports a cheap and easy method for obtaining pectin degrading bacteria from fruit waste. The carrot waste can be employed for the extraction of pectinase enzyme which is having numerous industrial applications and in a cheaper manner.

Acknowledgements

We pay special thanks to Ms Tisha Patel and our faculty members for their support to encourage and complete this study well and to the Department of Microbiology and Biotechnology, School of Sciences, Gujarat University, Ahmedabad.

References

Aaisha, G. A., & Barate, D. L. (2016). Isolation and identification of pectinolytic bacteria from soil samples of Akola region, India. *Int J Curr Microbiol App Sci*, *5*, 514-521.

Bhardwaj, V., & Garg, N. UTILIZATION OF FRUITS WASTE FOR PECTINASE PRODUCTION.

Garg, G., Singh, A., Kaur, A., Singh, R., Kaur, J., & Mahajan, R. (2016). Microbial pectinases: an ecofriendly tool of nature for industries. *3 Biotech*, *6*(1), 1-13.

Kashyap, D. R., Vohra, P. K., Chopra, S., & Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. *Bioresource technology*, *77*(3), 215-227.

Kumar, A., & Sharma, R. (2012). Production of alkaline pectinase by bacteria (Cocci sps.) isolated from decomposing fruit materials. *Journal of Phytology*, *4*(1).

Oliyad, J. (2015). *Pectinase from Bacillus Subtilis Strain Btk27: Optimization of Cultural Conditions, Characterization of the Enzyme and its Potential Application in Coffee Processing* (Doctoral dissertation, Addis Ababa University).

Patil, R. C., Murugkar, T. P., & Shaikh, S. A. (2012). Extraction of pectinase from pectinolytic bacteria isolated from carrot waste. *International Journal of Pharma and Bio Sciences*, *3*, B261-B266.

Priya, V., & Sashi, V. (2014). Pectinase enzyme producing Microorganisms. *International Journal of Scientific and Research Publications*, *4*(3), 2250-3153.

Sandhya, R., & Kurup, G. (2013). Screening and isolation of pectinase from fruit and vegetable wastes and the use of orange waste as a substrate for pectinase production. *Int Res J Biol Sci*, *2*(9), 34-39.

Sridevi, K., SivaRaagini, P., Sumanth, M., & Vijalakshmi, K. (2015). ISOLATION AND SCREENING OF PECTINOLYTIC BACTERIA FROM THE MANGO FRUIT YARDS.

Tapre, A. R., & Jain, R. K. (2014). Pectinases: enzymes for fruit processing industry. *International Food Research Journal*, *21*(2), 447-453.

Tariq, A., & Latif, Z. (2012). Isolation and biochemical characterization of bacterial isolates producing different levels of polygalacturonases from various sources. *African Journal of Microbiology Research*, *6*(45), 7259-7264.

Wakil, S. M., & Oyinlola, K. A. (2011). Diversity of pectinolytic bacteria causing soft rot disease of vegetables in Ibadan, Nigeria. *Journal of Applied Biosciences*, *38*, 2540-2550.

❑